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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/62, 5/10, 1/21, 1/19, A61K 39/21, 39/145, C07K 13/00, A61K 39/155, 39/00

A1

(11) International Publication Number: WO 94/24290

(43) International Publication Date: 27 October 1994 (27.10.94)

(21) International Application Number: PCT/GB94/00755

(22) International Filing Date: 8 April 1994 (08.04.94)

(30) Priority Data:
9307371.6 8 April 1993 (08.04.93) GB

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(81) Designated States: AU, BR, CA, CN, CZ, DE, FI, GB, HU, JP, KR, NO, NZ, PL, RU, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: FUSION PROTEINS BETWEEN ANTIGENIC AMINO ACID SEQUENCES AND BETA-2-MICROGLOBULIN

(57) Abstract

A hybrid fusion protein comprising a first antigenic amino acid sequence fused to a second amino acid sequence substantially homologous to B2M or a fragment thereof.

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FUSION PROTEINS BETWEEN ANTIGENIC AMINO ACID SEQUENCES AND BETA-2-MICROGLOBULIN

This invention concerns novel hybrid beta-2 microglobulin fusion proteins. It also relates to nucleic acid (DNA and RNA) coding for all or part of such proteins, and to their preparation. Pharmaceutical compositions containing such fusion proteins are useful both as components of prophylactic vaccines and as immuno-therapeutics for the post-exposure treatment of viral infection and malignancy.

Immune responses have two major components, humoral and cell-mediated. The first comprises antibodies, produced by B-lymphocytes, which bind to specific antigens and which serve a number of important functions such as neutralisation of viruses, complement fixation and immune complex formation. Antibody production is dependent on helper T cells, which mediate the antigen specific recognition of peptide epitopes displayed by Class II Major Histocompatibility Complex (MHC) molecules on the surface of antigen presenting cells.

The second arm of the immune system involves effector T cells, in particular cytotoxic T lymphocytes (CTLs) which can recognise and kill infected or transformed cells. Such responses are particularly important in the defense against and immunity from viral infections, though they are also involved in host responses against certain tumours. CTL responses are also antigen specific, involving subsets of CD8 positive T cells. Immune recognition is mediated by the T cell receptor which recognises the peptide antigen displayed on Class I MHC molecules.

Class I MHC molecules are ubiquitously expressed and consist of two polypeptide chains. The first is an alpha chain of about 45kDa comprising 3 domains. Two of these domains bind to peptides derived from processing endogenously synthesised proteins, such as viral components, and present them to the T cell receptor. These two domains are linked to a single membrane spanning anchor region by a third, immunoglobulin-like domain. The second component is beta-2 microglobulin (B2M), a 100 amino acid protein which can exist free in the serum as well as a part of Class I MHC. The two chains normally associate in the ER, along with peptides produced by the degradation of endogenous proteins to form a

ternary complex which is displayed on the cell surface. Although binary complexes lacking peptide can be formed, these are unstable and are normally recycled or degraded (Ljungren et al. Nature 346, 476- (1990).

Crystallographic data reveals that the antigenic peptide binds to a groove between the first two domains of the alpha subunit, the base of which is formed from strands of beta sheet, and the walls from two alpha helices (Bjorkman et al. Nature 329, 506-512 (1987)). The nature of the side chains in this region of the alpha chain are critical in determining the peptide selectivity of the molecule, and hence the ability of different Class I MHC alleles to respond to particular epitopes (Bjorkman et al. Nature 329, 512-518 (1987)). Although it is the alpha subunit that binds the peptide, B2M plays an essential role in allowing binding, presumably by stabilising the ternary complex (Vitiello et al. Science 250, 1423-1426 (1990); Rock et al. PNAS 88, 310-304 (1991)). While some free alpha subunit can reach the cell surface in the absence of B2M, and can bind and present peptide, it is considerably less efficient than in the presence of B2M (Bix and Raulet. Exp. Med. 176, 829-834 (1992).

Although the normal route of antigen presentation by Class I MHC involves the degradation of endogenously synthesised proteins, it is possible to achieve antigen presentation by addition of high concentrations of peptide to antigen presenting cells (APC) *in vitro*. The use of peptides as immunogens has a number of potential advantages, the most significant of which are that it avoids the use of inactivated or attenuated viral particles, and that small peptides can be synthesised chemically, avoiding the requirement for biological production routes. The most efficient presentation is obtained with peptides of from 9-11 residues in length, which corresponds to the length of the groove if they are in an extended conformation.

However, there are major problems associated with this method of eliciting a CTL response. Although such a use of peptides is adequate for demonstrating immune responses such as T cell-mediated killing *in vitro*, it is not an efficient process, and does not offer a practical route to *in vivo* immunisation. CTL responses may be induced by immunisation with certain small peptide fragments but such peptides

may elicit poor antibody responses. Further, in an outbred population, individuals of different MHC class I and class II haplotypes will respond to different peptide epitopes; many peptides may be required to prime all the possible haplotypes.

Most of the available evidence also indicates that immunisation with whole proteins does not give rise to a CTL response. Such CTL responses that have been induced require the presence of powerful adjuvants or live vector which have not been licensed for use in humans. For example, recombinant HIV gp160 presented in ISCOMs, but not in FCA, FIA or buffered saline, induced HIV-1 envelope-specific CTL activity in BALB/c mice. It is generally felt that approval for the use of ISCOMs in humans is unlikely to be forthcoming. Recombinant vaccinia viruses expressing the V3 region of the HIV envelope gp160 also induce specific CTL responses to the V3 region, but immunisation with vaccinia has raised concerns both about safety for immunocompromised patients, and about efficacy. A simpler and more efficient way to induce CTL responses is therefore an important goal, and has been sought actively for a number of years though with only limited success.

It has been shown that the exchange of B2M chains in MHC class I will occur in the presence of free B2M (Hayfil and Strominger, PNAS USA 76(11) 5834-5838 (1979)), and that the binding of exogenous peptides to these molecules occurs upon association and reassociation of B2M light chains (Rock et al., PNAS USA 87 7517-7521 (1990); Kozlowski et al. Nature 349, 74-77 (1991)). The presentation of exogenously added peptide can therefore be made more efficient by the addition of B2M. The use of exogenous B2M in this way to enhance immune responses against peptides *in vivo* is described in WO-A-91/16924.

However, there are a number of problems with this approach. The use of an admixture, while a convenient tool for *in vitro* use, is less appropriate for in vivo administration, due to the problems of polypharmacy for product registration.

Further, despite the DNA sequence of B2M being known, it has not been possible to produce recombinant B2M efficiently. B2M for use in the enhancement of immune responses would therefore need to be purified from natural sources such as serum or urine. Not only is this difficult and expensive, but blood products must

be screened for a number of contaminants before being acceptable for use in vaccines.

Despite the fact that interactions between the C-terminus of the peptide and the alpha subunit have been shown to be essential for peptide binding and presentation, the present invention has found that peptides fused to the N-terminus of B2M are still capable of binding to the groove in the alpha subunit. This binding is achieved without sacrificing the ability of the ternary complex to bind and trigger the T-cell receptor.

According to the first aspect of the invention there is provided a hybrid fusion protein comprising a first antigenic amino acid sequence fused to a second amino acid sequence substantially homologous with B2M or a fragment thereof.

Preferably the first antigenic sequence is fused to the B2M sequence via a short linker sequence, to span the gap between the peptide and the B2M with minimal disruption to the conformation of the light chain.

The use of B2M fusions offers considerable advantages. The binary complex between the alpha subunit and the B2M fusion is stabilised relative to the corresponding ternary complex, prolonging the ability of the peptide to stimulate the T cell receptor, thereby increasing the effective potency of the peptide. The problems of polypharmacy are avoided, simplifying the development of the compound. This approach also has a number of product registration advantages by minimising the number of proteins present in the vaccine, and may conform more closely to guidelines on quality and safety. Finally, only a single GMP production route is required. These latter considerations are particularly important since multiple epitopes might be required to produce a vaccine effective against the background of MHC diversity. A final advantage is that because the peptide is delivered efficiently to the MHC, the requirement for adjuvants may be obviated.

The expression "substantially homologous", when describing the relationship of an amino acid sequence to a natural protein, means that the amino acid sequence can be identical to the natural protein or can be an effective but truncated or

modified form of the natural protein. As a practical matter, though, most analogues will have a high degree of homology with the prototype molecule if biological activity is to be substantially preserved. It will be realised that the nature of changes from the prototype molecule is more important than the amount of them. As guidance, though, at the amino acid level, it may be that at least 60%, 70%, 80%, 90%, 95% or even 99% of the residues will be the same as the prototype molecule; at the nucleic acid level, nucleic acid coding for an analogue may for example hybridise under stringent conditions (such as at approximately 35°C to 65°C in a salt solution of approximately 0.9 molar) to nucleic acid coding for the prototype molecule, or would do so but for the degeneracy of the genetic code.

Fragments of B2M for use in this invention will retain the ability to augment immune responses to the peptides to which they are fused. Preferably at least 60%, 70%, 80%, 90%, 95% or even 99% of the residues will be retained.

Proteins substantially homologous to B2M include naturally occurring B2M and modified B2M, a variant found in connection with a variety of types of cancer and disorders of the immune system.

The antigenic sequence may correspond to a sequence derived from or associated with an aetiological agent or a tumour. The aetiological agent may be a microorganism such as a virus, bacterium, fungus or parasite. The virus may be: a retrovirus, such as HIV-1, HIV-2, HTLV-I, HTLV-II, HTLV-III, SIV, BIV, LAV, ELAV, CIAV, murine leukaemia virus, Moloney murine leukaemia virus, and feline leukaemia virus; an orthomyxovirus, such as influenza A or B; a paramyxovirus, such as parainfluenza virus, mumps, measles, RSV and Sendai virus; a papovavirus, such as HPV; an arenavirus, such as LCMV of humans or mice; a hepadnavirus, such as Hepatitis B virus; a herpes virus, such as HSV, VZV, CMV, or EBV. The tumour-associated or derived antigen may for example be a proteinaceous human tumour antigen, such as a melanoma-associated antigen, or an epithelial-tumour associated antigen such as from breast or colon carcinoma.

The antigenic sequence may be also derived from a bacterium, such as of the genus *Neisseria*, *Campilobacter*, *Bordetella*, *Listeria*, *Mycobacteria* or *Leishmania*,

or a parasite, such as from the genus *Trypanosoma, Schizosoma, Plasmodium*, especially *P. falciparum*, or from a fungus, such as from the genus *Candida, Aspergillus, Cryptococcus, Histoplasma* or *Blastomyces*.

Preferred antigenic sequences correspond to CTL epitopes from a retrovirus, a paramyxovirus, an arenavirus or a hepadna virus, or a human tumour cell. Examples include epitopes from:

1) HIV (particularly HIV-1) gp120,
2) HIV (particularly HIV-1) p24
3) VZV gpI, gpII and gpIII
4) LCMV nucleoprotein,
5) Influenza virus nucleoprotein,
6) HPV L1 and L2 proteins,
7) Human papilloma virus E5 and E7
8) Malaria CSP or RESA antigens,
9) Mycobacterium p6,
10) GA 733-2 epithelial tumour-associated antigen,
11) MUC-1 repeat sequence from epithelial tumour-associated antigen,
12) Melanoma MZ2-E antigens
13) Melanoma p97 associated antigen,

Particularly preferred antigenic sequences are derived from the third variable domain of an envelope protein of a lentivirus. This region of lentiviruses, known as the V3 loop or GPGR loop is found between amino acids 300 and 330 of gp120 of HIV-1 and in analogous positions of other lentiviruses. The V3 loop is, for HIV-1 at least, the major neutralising epitope of the virus (Putney *et al* 1986 *Science* 234, 1392; Rusche *et al* 1988 *Proc. Natl. Acad. Sci.* 85, 3198; Palker *et al* 1988 *Proc. Natl. Acad. Sci.* 85 1932; and Goudsmit *et al.*, 1988 *AIDS* 2 157). The antigenic portion of choice may constitute the whole of the V3 loop when derived from different strains. However, multiple copies of a conserved sequence of the V3 loop may be useful in conferring immunity against more than one isolate of a virus (such as HIV-1).

B2M fusion proteins in accordance with the invention can in principle be made by any convenient method including coupling successive amino acid residues together, or by the chemical coupling of two or more oligo- or polypeptide chains or of existing (for example natural) proteins. Although proteins may in principle be synthesised wholly or partly by chemical means, the route of choice will be ribosomal translation, preferably *in vivo*, of a corresponding nucleic acid sequence.

According to a second aspect of the invention, therefore, there is provided nucleic acid coding for a fusion protein as described above. Both DNA and RNA are within the scope of the invention. DNA may be chemically synthesised and/or recombinant.

Recombinant DNA in accordance with the invention may be in the form of a vector. The vector may for example be a plasmid, cosmid or phage. Vectors will frequently include one or more selectable markers to enable the selection of cells transformed (or transfected: the terms are used interchangeably in this specification) with them and, preferably, to enable selection of cells harbouring vectors incorporating heterologous DNA. Appropriate translational initiating and termination signals will generally be present. Additionally, if the vector is intended for expression, sufficient transcriptional regulatory sequences to drive expression will be included. Vectors not including regulatory sequences are useful as cloning vectors. According to a third aspect of the invention there is provided a vector including nucleic acid as described above. It is to be understood that the term "vector" is used in this specification in a functional sense and is not to be construed as necessarily being limited to a single nucleic acid molecule.

Expression vectors in accordance with the invention will usually contain a promoter. The nature of the promoter will depend upon the intended host expression cell. For yeast, *PGK* is a preferred promoter, but any other suitable promoter may be used if necessary or desirable. Examples include *GAP*D, *GAL*1-10, *PH05*, *ADH*1, *CYC*1, Ty delta sequence, *PYK* and hybrid promoters made from components from more than one promoter (such as those listed). For insect cells, a preferred promoter is the polyhedrin promoter from *Autographica californica* nuclear polyhedrosis virus (AcNPV). Those skilled in the art will be able to

determine other appropriate promoters adapted for expression in these or other cells. Vectors not including promoters may be useful as cloning vectors, rather than expression vectors.

Cloning vectors can be introduced into *E. coli* or any other suitable hosts which facilitate their manipulation. Expression vectors may be adapted for prokaryotic expression in bacterial cells, such as *E. coli*,. However, for preference vectors are adapted for expression in a microbial eukaryotic cell, such as a yeast (including but not limited to *Saccharomyces cerevisiae* and *Pichia pastoris*) or a higher eukaryotic cell such as insect cell lines such as *Spodoptera frugiperda* SF9, or mammalian cells including Chinese hamster ovary (CHO) cells, mouse myeloma cell lines such as P3X63-Ag8.653, COS cells, HeLa cells, BHK cells, melanoma cell lines such as the Bowes cell line, mouse L cells, human hepatoma cell lines such as Hep G2, mouse fibroblasts and mouse NIH 3T3 cells. Performance of the invention is neither dependent on nor limited to any particular strain of microorganism or cell type: those suitable for use with the invention will be apparent to those skilled in the art, following the teaching of this specification. According to a fourth aspect of the invention there is provided a host cell transfected or transformed with DNA described above.

Despite the DNA sequence of B2M being known, to date it has not been possible to produce recombinant B2M efficiently. Extremely low yields are produced by expression in mammalian cells, and the protein is incorrectly folded following expression in *E. coli.* Nor are yeasts a universally host for the purpose. Standard techniques for obtaining expression of B2M in perhaps the most widely used yeast for such purposes, namely *S. cerevisiae*, resulted in very poor yields.

The *Pichia pastoris* expression system is well known, and has particular advantages in its ease of scalability for large scale production. High level expression has been obtained for a number of proteins in that host, but equally, some have proved difficult to produce. There is no obvious correlation between the properties of a particular polypeptide and its ability to be highly expressed in the *Pichia* system.

In contrast with the low level expression of B2M in *S. cerevisiae*, reasonable yields of the fusion proteins of this invention can be obtained in the *Pichia* system.

Therefore, the invention includes a method of producing fusion proteins of the invention by cultivating a methylotropic yeast harbouring an expression vector comprising DNA encoding the relevant fusion protein, and recovering the expressed fusion protein.

Methylotropic yeast strains include *Pichia*, in particular *P. pastoris, Hansenula, Candida* and *Torulopsis.* Use of *Pichia pastoris* is presently preferred.

Recombinant DNA encoding the fusion proteins of the invention may be incorporated in a yeast expression vector for expression in methylotropic yeast in accordance with the invention. Such vectors will usually contain a promoter. *AOX* is a preferred promoter, but any other suitable promoter may be used if necessary or desirable. Examples include *GAP*D, *GAL*1-10, *PH05*, *ADH*1, *PGK*, *CYC*1, Ty delta sequence, *PYK* and hybrid promoters made from components from more than one promoter (such as those listed).

To obtain secretion of the fusion protein from the yeast cells after expression, the expression vector preferably includes a secretion leader sequence fused to the B2M sequence of the hybrid fusion protein. After secretion, the leader sequence is automatically cleaved from the B2M protein by enzyme(s) produced naturally during the cultivation of the transformed yeast cells. Such secretion techniques are well known. Secretion leaders known to the art include the alpha factor, Pho1, HSA and Suc2. If cleavage is not 100% accurate, the final yield of fusion protein may be contaminated with a fusion of B2M/epitope hybrid with part of the secretion factor sequence, indicating incomplete removal of the secretion factor leader. Although the hybrid protein of the invention may be separated from the contaminant by standard purification methods, for example those based on differing molecular weights, it would be desirable to avoid the difficulty if possible.

It appears the Pho1 leader is in general correctly cleaved from the hybrid fusion proteins of the invention, and it is therefore the presently preferred secretion factor

for use in accordance with the invention.

The B2M fusion proteins of the first aspect are useful as vaccines. These might be used prior to exposure as prophylactic agents, or after exposure as immunotherapeutic agents to enhance the clearance of viral infection. Such fusion proteins would be administered by conventional routes, either i.v., i.m. or s.c., though the s.c. route may be preferred.

According to a fifth aspect of the invention, there is provided a pharmaceutical or veterinary formulation comprising a compound of general formula I and a pharmaceutically or veterinarily acceptable carrier. One or more fusion proteins of the first aspect may be present in association with one or more non-toxic pharmaceutically or veterinarily acceptable carriers such as sterile physiological saline or sterile PBS, and/or diluents and/or adjuvants and if desired other active ingredients. Sterility will generally be essential for parenterally administrable vaccines. One or more appropriate adjuvants may also be present. Examples of suitable adjuvants include muramyl peptide compounds such as prototype muramyl dipeptide, aluminium hydroxide and saponin. Coadministration with cytokines may also be considered, eg IFNγ may potentiate the immune response by inducing class I MHC expression.

It may be preferred that, when used as prophylactic vaccines, the B2M agents are used in combination with subunit vaccines designed to induce good neutralising antibody responses. However, this may not be necessary as there is some evidence that CTL responses alone can protect against infection.

According to a sixth aspect of the invention, there is provided a process for the preparation of a pharmaceutical or veterinary formulation in accordance with the fifth aspect, the process comprising admixing a B2M fusion protein of the first aspect and a pharmaceutically or veterinarily acceptable carrier.

The active ingredient may be administered parenterally in a sterile medium. Depending on the vehicle and concentration used, the drug can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as a local

anaesthetic, preservative and buffering agents can be dissolved in the vehicle.

Particulate antigens produced in accordance with the invention may be useful in the preparation of vaccines, for example immunotherapeutic vaccines, which form a further aspect of the invention.

The following Example illustrate the invention:

Figure 1 shows the yeast expression vector pSW6, into which is cloned the B2M gene according to example 2.

Figure 2 shows the *Pichia* expression vector pHILD1 used as the starting point in example 3 for the construction of the expression vector for high-level expression of B2M in *P.pastoris,* using the alpha factor leader sequence.

Figure 3 shows the intermediate vector pLHD4 used in example 3 in the construction of the *P. pastoris* alpha factor/B2M expression vector.

Figure 4 shows the *P. pastoris* alpha factor/B2M expression vector pLH15 prepared according to example 3.

Figure 5 shows the Pichia expression vector pHILS1 used as the starting point in example 4 for the construction of the expression vector for high-level expression of B2M in *P.pastoris,* using the Pho1 leader sequence, and the manipulations leading to the final P. pastoris Pho1/B2M expression vector pLH46 according to example 4.

Figure 6 shows the the sequence of the Pho1/B2M fusion included in pLH46 (Figure 5), where the underlined sequence represents the cloning site, the italics represent the Pho1 signal sequence, and the bold letters are identical to the sequence of the B2M gene.

Figure 7 shows the resut of the test described in Example 9.

Example 1 - Isolation of the B2M gene and preparation for cloning

RNA was purified from human blood cells using the isothiocyanate guanidinium method (RNAzol B™, Biogenesys). This RNA was used as the template for synthesis of first strand cDNA with the use of oligo dT primers using the method of Maniatis (in Molecular Cloning, 1989).

The cDNA was then amplified in PCR reactions using primer (5'-GACAAGCTTGGATAAAAGAATCCAGCGTACTCCAAAG-3') SEQ ID 1 to add a *Hind*III restriction site and an adaptor sequence encoding the last 5 amino acids of the *S.cerevisiae* alpha mating factor at the 5' end of the gene, and (5'-CATAGGATCCTTATTACATGTCTCGATCCCACTT-3') SEQ ID 2 that added a termination codon and a *Bam*H1 site at the 3' end of the gene. PCR products were generated in 30 cycles of denaturation at 97°C for 1 min, annealing at 60°C for 1 min and elongation 72°C for 30 seconds. The reactions were carried out in 1 mM MgCl2, 50mM KCl, 10mM Tris pH 8.3 using 50ng of primer in a volume of 50µl. The sequence of the gene is shown in Figure 2. After PCR amplification the DNA was digested with *Hind*III and *Bam*H1 and ligated into *Hind*III-*Bam*HI calf intestinal phosphatase-treated M13mp19. M13 clones were then sequenced to identify isolates that had no difference to the published sequence using universal primer (5'-GTTTTCCCAGTCACGAC-3') SEQ ID 3. DNA was then purified from one of these clones and subcloned into the *S.cerevisiae* expression vector pSW6 (see Example 2).

Example 2 - Cloning the beta-2-microglobulin gene into the *S.cerevisiae* expression vector pSW6

An expression vector was designed to enable secretion of B2 microglobulin to the extracellular medium after expression in *S. cerevisiae*. Secretion aids purification and rapid analysis of B2M. The secretion signals from the yeast mating type factor alpha were used to direct export of the B2 microglobulin protein.

The yeast expression vector pSW6 (Figure 1) is based on the 2 micron circle from *S. cerevisiae*. (pSW6 is deposited in *S. cerevisiae* strain BJ2168 at the National

Collection of Industrial and Marine Bacteria Limited, 23 St. Machar Drive, Aberdeen AB2 1RY, United Kingdom under Accession No. NCIMB 40326). pSW6 is a shuttle vector capable of replication in both *E. coli* and *S. cerevisiae* and contains an origin of DNA replication for both organisms, the *leu2* gene (a selectable marker for plasmid maintenance in the yeast host) and the ampicillin resistance locus for selection of plasmid maintenance in *E. coli*. (The DNA sequence for the vector is disclosed in WO-A-09125). The ability to passage this vector through *E. coli* greatly facilitates its genetic manipulation and ease of purification. pSW6 contains an alpha-factor pre-pro-peptide gene fused in-frame to a gene encoding human epidermal growth factor (EGF). The expression of this fusion is under the control of an efficient galactose regulated promoter which contains hybrid DNA sequences from the *S. cerevisiae* GAL 1-10 and phosophoglycerate kinase (PGK) promoters. Transcription of the EGF gene is terminated in this vector by the natural yeast PGK terminator. The EGF gene in pSW6 can be removed by digestion with restriction endonucleases *Hin*dIII and *Bam*HI. This removes DNA encoding both EGF and 5 amino acids from the C-terminus of the alpha-factor pro-peptide. Genes to be inserted into the pSW6 expression vector must therefore have the general composition: *Hin*dIII site-alpha factor adaptor-gene-*Bam*HI site. The B2M gene prepared in Example 1 has this general composition.

After digestion with *Hin*dIII and *Bam*HI endonucleases, the pSW6 vector contains the alpha factor gene minus the codons for the last five amino acid residues. The B2M gene prepared as in Example 1 was then cloned into the pSW6 vector. The resulting vector was then transformed into the host strain BJ2168 using the method of Ito *et al* J. Bacteriol. (1983) **153** 163-168.

Example 3 - Construction of alpha factor/B2M *Pichia pastoris* Expression Vector

The expression vectors used for this work were derived from the Phillips Petroleum expression vector pHILD1.

Pichia expression vector pHILD1 (Figure 2) is a shuttle vector capable of

propagation in both *E. coli* (for ease of genetic manipulation) and in the methylotrophic yeast *Pichia pastoris*. pHILD1 can be obtained under licence from the Phillips Petroleum Company, Bartlesville Oklahoma, USA. The vector comprises sequences derived from the *E.coli* vector pBR322 and sequences derived from the genome of *Pichia pastoris*. The essential features are the 5' region of the *Pichia* AOX gene including the regulatable AOX promoter for high level transcription, the 3' region from the AOX gene containing the alcohol oxidase transcriptional terminator sequence, a further region from the 3' part of the ~AOX gene is included which together with the 5' AOX region is required for site directed integration of the expression cassette into the host genome. The *P. pastoris* histidinol dehydrogenase gene HIS4 is carried and used to complement the defective HIS 4 gene in *Pichia* host strains. The ampicillin resistance gene is carried to enable selection in the *E.coli* hosts used during genetic manipulation.

The pHILD1 vector was manipulated to allow expression of the B2M gene under the control of the alpha factor secretion signal. pHILD1 does not carry any sequences encoding secretion signals to allow export of heterologous proteins. To include such a signal, the vector was manipulated by the addition of sequences from the *S.cerevisiae* alpha factor leader. The vector was further engineered to provide a more optimal promoter context and to remove undesirable *Hind* III restriction sites which may interfere with the cloning of the B2M gene, a *Bam* HI site was then introduced 3' to the remaining *Hind* III to allow cloning of the B2M gene on a *Hind*III - *Bam*HI restriction site and to include a kanamycin resistance cassette enabling the selection of multicopy integrants in transformed *Pichia* host strains. The stages of the manipulations are shown below.

1) Inclusion of alpha factor secretion signals

The alpha factor sequences were cloned into the pHILD1 vector from the *S. cerevisiae* expression vector pSW6 (Figure 1) (see example 2 for details). The alpha factor sequences were isolated from pSW6 on ca 430 bp *Bgl* II-*Bam* HI DNA fragment, this fragment contains the alpha factor sequences fused to a human epidermal growth factor synthetic gene (EGF). The overhanging ends of this DNA fragment were first filled in using klenow fragment of *E.coli* DNA polymerase I

together with the required deoxynucleoside triphosphates according to standard methodology. The flush ended fragment was then cloned into the pHILD1 vector that had been treated with *Eco* RI and then blunt ended as above. The integrity of the resultant plasmid pLH001 was checked by a combination of restiction digestion and DNA sequence analysis. The primer use for sequence analysis was (5'-GCATTCTGACATCCTCT-3'), SEQ ID 4. The sequence of the alpha factor fusion was confirmed.

2) Mutagenesis to optimise vector for B2M expression

To remove unwanted *Hind* III restriction sites, optimise the promoter region and introduce a *Bam* H1 site from the pLHOO1 vector, relevant fragments were cloned into bacteriophage M13 for site directed mutagenesis. The fragments cloned, the primers used for mutagenesis, and the primers used for sequencing are detailed below. Furthermore, a kanamycin resistance cassette was modified for introduction into the final expression vector to allow selection for muticopy integrants when the vector is introduced into *Pichia* host strains.

A 1220 bp *Sac*I-*Sac*I fragment was isolated from pLH001 and cloned into M13 mp19. This M13 construct was then used for mutagenesis in which a *Hind* III site was removed using oligonucleotide primer (5'-CGTTAAAATCAACAACTTGTCAATTG-GAACC-3'), SEQ ID 5, the mutants were identified by sequence analysis with sequencing primer (5'-GGAAATCTCACAGATCT-3'), SEQ ID 6. This fragment was further modified by deletion mutagenesis to optimise the 5' untranslated leader region preceeding the AOX promoter, which is now identical to that found in the natural 5' untranslated leader of the AOX1 gene on the *Pichia* genome. Having the correct context around the 5' untranslated leader is preferred for maximal expression. The mutagenesis primer used for this step was (5'GAAGGAAATCTCATCGTTTCGAATA-3'), SEQ ID 7. The mutant was identified by sequence analysis with sequencing primer (5'-GCTAATGCGG-AGGATGC-3'), SEQ ID 8.

Two further *Hind* III sites was removed from from the 770bp *Sac* I-*Xba* I fragment of pLH001 by mutagenesis. The *Sac*I-*Xba*I fragment of pLH001 was first cloned into

M13 mp18 and one of the *Hind* III sites was removed using the primer (5'-CCGGCATTACAACTTATCGATAAGCTTGCAC-3'), SEQ ID 9. The identity of this mutant was confirmed by sequence analysis using the sequencing primer (5'-GCGCATTGTTAGATTTC-3'), SEQ ID 10. A second *Hind*III site was removed from this newly mutagenised fragment using mutagenesis primer (5'-CTTATCGATCAACTTGCACAAACG-3'), SEQ ID 11. The correct mutant was identified by sequence analysis using sequence primer SEQ ID 9 (see above).

Before reassembly, a *Bam* HI site was introduced into the *Hind* III deleted *Sac* I-*Xba* I fragment to allow subsequent cloning of the B2M gene of example 1 on a *Hind* III-*Bam* HI fragment. The mutagenesis primer used to introduce the *Bam* H1 site was (5'-GTCATGTCTAAGGCGGATCCTTATTAAC-3'), SEQ ID 12. The identity of the mutant was identified using sequencing primer (5'-GCATTCTGACATCCTCT-3') SEQ ID 13.

3) Modification of the Kanamycin resistance cassette

A kanamycin resistance cassette was purchased from Pharmacia Biosystems Limited, Davy Avenue. Knowlhill, Milton Keynes, MK5 8PH. Great Britain. This cassette is supplied as an EcoRI fragment by Pharmacia and this was cloned into M13 mp19 as an *Eco*R1 fragment. The internal *Hind* III was deleted using mutagenesis primer (5'- GAGAATGGCAACAACTTATGCATT-3'), SEQ ID 14. The mutation was confirmed using sequencing primer (5'-CCAACATCAATACAACC-3'), SEQ ID 15.

4) Reassembly of expression vector

The vector was reconstructed in a stepwise manner using the Phillips petroleum vector pHILD1 as a backbone for the cloning.

To rebuild the expression vector including the mutagenised fragments, the modified ca 770 bp *Sac*I-*Xba*I fragment was first ligated into *Sac*I -*Xba*I treated pHILD1 vector. The integrity of the recombinant construct was then confirmed by restriction analysis and DNA sequence analysis using the oligonucleotide

sequencing primer (5'- GCGCATTGTTAGATTTC - 3'), SEQ ID 16, the construct was called intermediary vector 1. The modified *Sac* I-*Sac* I fragment was next cloned into intermediary vector 1 which had been treated with *Sac* I and calf intestinal phosphatase. The resultant construct named intermediary vector 2, was again confirmed by restriction analysis and DNA sequence analysis with oligonucleotide primers (5'-GGAAATCTCATAGATCT-3'), SEQ ID 17 to read through the deleted *Hind* III site and (5'-GCTAATGCGGAGGATGC-3'), SEQ ID 18 to read through the optimised 5' untranslated leader region. Intermediary vector 2 is a homologue of pHILD1 which lacks the unwanted *Hind* III sites, has an optimised 5' untranslated region, contains sequences encoding the *S. cerevisiae* alpha factor secretion signals followed by the remaining *Hind* III site and which has a *Bam* HI site 3' to the *Hind*III site to allow cloning of the B2M gene.

A 1200bp *Hinc*11 fragment containing the mutagenised kanamycin cassette was removed from the M13 mp19 mutagenesis vector (used to remove the *Hind* III site from the kanamycin resistance gene) and cloned into the unique *Nae* 1 site of the intermediary vector 2. The vector was renamed pLHD4. The integrity of pLHD4 was confirmed by restriction analysis. A map of pLHD4 is shown in Figure 3. pLHD4 contains the human EGF gene fused to the *S. cerevisiae* secretion signal.

5) Construction of the B2M Pichia expression vector

The final expression vector for B2 microglobulin expression was constructed by cloning the *Hind* III - *Bam* HI fragment of example 2 into pLHD4. (This 320bp*Hind* III-*Bam* HI fragment contains the B2M gene fused to the 3' end of a sequence encoding the 5 amino acids of the yeast alpha factor which precede the KEX2 cleavage site required for liberation of the mature peptide following secretion from the *Pichia* host).

The *Hind* III-*Bam* H1 fragment was obtained by restriction digestion of the *S. cerevisiae* expression vector pSW6 B2M. This fragment was purified on a 1.5% low melting temperature agarose gel then ligated to *Hind* III-*Bam* H1, calf intestinal phosphatase treated pLHD4. The resultant recombinant was called pLH15. The vector is shown on Figure 4. (At this time the Kanamycin resistance gene was

defective, due to a then unknown deletion of bases, which occured during mutagenesis to delete the internal *Hindl* III site. A ca 2070 bp *Sac* I- *Xba* I fragment was removed from the resultant construct and cloned into the expression vector pHILD4, so conferring Kanamycin resistance) The integrity of the construct was confirmed by restriction analysis and sequencing analysis using the sequencing primer (5'-GCATTCTGACATCCTCT -3'), SEQ ID 19.

Example 4 - Construction of Pho1/B2M *Pichia pastoris* Expression Vector

The expression vectors used for this work were derived from the Phillips Petroleum expression vector pHILS1 (Figure 5)

The starting expression vector for manipulation was the *Pichia pastoris* shuttle vector pHILSI (obtainable under licence from Phillips Petroleum) capable of propagation in both *E. coli* (for ease of genetic manipulation) and in the methylotropic yeast *Pichia pastoris*. It contains the Pho1 leader sequence from the *Pichia pastoris* acid phosphatase gene. It also contains sequences from the *E. coli* vector pBR322 and sequences derived from the genome of *Pichia pastoris*. The essential features are the 5' region of the AOX gene including the methanol regulatable AOX promoter for high level transcription, the 3' region from the AOX gene containing the alcohol oxidase transcriptional terminator sequence, a further region from the 3' region which together with the 5'AOX region is required for site directed integration into the expression cassette into the chromosome. The *Pichia* histidinol dehydrogenase gene HIS4 is carried and used to complement the defective copy of the his4 gene on the host genome. The ampicillin resistance gene is carried to enable selection in the *E. coli* host used during the genetic manipulation.

The pHILS1 vector was manipulated to allow expression of the B2M gene under the control of the Pho1 secretion signal:

The gene encoding B2M was cloned into the pHILSI vector from the *S. cerevisiae* expression vector pSW6 containing the gene as a *Hind*III-*Bam*HI fragment

(Example 2). The overhanging end of the DNA fragment at the *Hind*III site was first filled in to create a blunt end using the Klenow fragment of DNA polymerase I together with the required deoxynucleotide triphosphates according to standard methodology. The vector pHILSI was restricted with restriction endonucleases *Eco*R1 and *Bam* H1. The overhanging end of the DNA fragment at the *Eco*R1 site was filled in as described above to create a blunt end. The B2M gene, as a blunt end *Bam*HI site was cloned into the blunt end *Bam*HI treated vector. The integrity of the resultant vector, intermediary vector 3, was confirmed by restriction analysis.

The *Hind*III-*Bam*HI fragment from the expression vector pSW6 contains the B2M gene fused at the 3' end to the last 5 amino acids of the yeast alpha factor which precedes the KEX2 cleavage site required for liberation of the mature peptide following secretion from the *Pichia pastoris* host when using the alpha factor sequence. This sequence and a few bases at the cloning site were deleted to allow the direct fusion of the Pho1 leader sequence and the B2M gene. The sequence deleted is (5'-CGAGAATTAGCTTGGATAAAAGA-3'), SEQ ID 20.

A ca 1150 bp (*Sst*I) *Sac*I-*Bam*HI fragment was isolated from the intermediary vector 3 and cloned into the vector M13mp18. This fragment contains 700bp of the 5' AOX promoter sequence, Pho1 signal sequence and the B2M gene. The M13 construct was then used for mutagenesis to delete the intervening sequence between the Pho1 sequence and the B2M gene. The oligonucleotide primer used for mutagenesis was (5'-GGAGTACGCTGGATAGCGAAGACAGATTG-3') SEQ ID 21. The mutants were identified by sequence analysis using the sequencing primer (5'-CCATTCTCTGCTGGATG-3') SEQ ID 22. The sequence of Pho1/B2M is shown in Figure 6, SEQ ID 23

The vector was reconstructed using the expression vector pLH15 (Example 3(5)) as a backbone for the cloning. The vector was restricted with the restriction endonucleases (*Sst*) *Sac*I and *Bam*HI to release a ca 1150bp fragment containing a 700bp fragment of the 5' AOX promoter sequence and alpha factor signal sequence fused to the B2M gene, which was then replaced by the mutagenised (*Sst*) *Sac*I-*Bam*HI fragment containing a 700bp fragment of the 5' AOX promoter sequence, Pho1 signal sequence fused to the B2M gene. The resultant

recombinant was called pLH46. The vector is shown on Figure 6. The integrity of the construct was confirmed by restriction analysis using the sequencing primer (5'-GCATTCTGACATCCTCT-3') SEQ ID 24.

Example 5 - Construction of vectors pGC633 containing the DNA sequence of the Sendai virus epitope

The expression vector pGC633 contains the gene encoding B2M fused at the 5' end to the Sendai virus epitope (Kast et al Proc. Natl. Acad. Sci. 88, 2283-2287 (1991)) via a short linker. The epitope/linker peptide sequence fused to the B2M sequence is FAPGNYPALGGGGG, SEQ ID 25. The underlined amino acids constitute the linker sequence, designed to span the gap between the peptide and the B2M with minimal disruption to the conformation of the light chain.

The expression vector pLH46 (Example 3) was restricted with the restriction endonucleases *Sac*I (*Sst*I) and *Bam*HI to release a ca 1150bp fragment containing a 700bp fragment of the 5' AOX promoter sequence, Pho1 signal sequence fused to the B2M gene. This fragment was cloned into M13mp18 which had been restricted with *Sac*I (*Sst*I) and *Bam*HI. The M13 construct was then used for mutagenesis to insert the sequence encoding the Sendai peptide. The oligonucleotide primer used for mutagenesis was (5'-GGAGTACGCTGGATACCACCACCACCACCCAAAGCTGGGTAGTTACCTGGAGC GAAAGCGAAGACAGATT-3'), SEQ ID 26. The mutants were identified by sequence analysis using sequencing primer (5'-CCATTCTCTGCTGGATG-3'), SEQ ID 27.

The vector was reconstructed using the expression vector pLH46 (Example 3) as a backbone for the cloning. The vector was restricted with the restriction endonucleases *Sac*I (*Sst*I) and *Bam*HI to release a ca 1150bp fragment containing a 700bp fragment of the 5' AOX promoter sequence, Pho1 signal sequence fused to the B2M gene, and replaced with the mutagenised fragment. The integrity of the construct was confirmed by restriction analysis and sequencing analysis using the sequencing primer (5'-GCATTCTGACATCCTCT-3'), SEQ ID 28.

Example 6 - Construction of vectors pGC638 containing the DNA sequence of the Influenza A virus epitope

the expression vector pGC638 contains the gene encoding B2M fused at the 5' end to the influenza virus epitope (Current Biology, 3 No.12 1993). The epitope/linker peptide sequence fused to the B2M sequence is GILGFVFTLGGGGGGSSS, SEQ ID 29. The underlined amino acids constitute the linker sequence, designed to span the gap between the peptide and the B2M with minimal disruption to the conformation of the light chain.

The expression vector pLH46 (Example 3) was restricted with the restriction endonucleases *Sac*I (*Sst*I) and *Bam*HI to release a ca 1150bp fragment containing a 700bp fragment of the 5' AOX promoter sequence, Pho1 signal sequence fused to the B2M gene. This fragment was cloned into M13mp18 which had been restricted with *Sac*I (*Sst*I) and *Bam*HI. The M13 construct was then used for mutagenesis to insert the sequence encoding the Sendai peptide. The oligonucleotide primer used for mutagenesis was (5'-GGAGTACGCTGGATAGAAGAAGAACCACCACCACCACCACCCAAAGTGAAAACGAAACCCAAAATACCAGCGAAGACAGATT-3'), SEQ ID 30. The mutants were identified by sequence analysis using sequencing primer (5'-CCATTCTCTGCTGGATG-3'), SEQ ID 27.

The vector was reconstructed using the expression vector pLH46 (Example 2) as a backbone for the cloning. The vector was restricted with the restriction endonucleases *Sac*I (*Sst*I) and *Bam*HI to release a ca 1150bp fragment containing a 700bp fragment of the 5' AOX promoter sequence, Pho1 signal sequence fused to the B2M gene, and replaced with the mutagenised fragment. The integrity of the construct was confirmed by restriction analysis and sequencing analysis using the sequencing primer (5'- GCATTCTGACATCCTCT-3'), SEQ ID 31.

Example 7 - Construction of *Pichia* expression strains

DNA of each of plasmids pGC633 and pGC638 (Examples 4 and 5) was linearised by cutting with the restriction endonuclease *Sac* I. This was to enable the

expression cassette to integrate via homologous recombination of sequences on the expression cassette and the host chromosome. In each case, the linearised plasmid was then transformed into *P.pastoris* strain GS115 (NRRL Y-1585) which has the genotype *his* 4. The use of this strain is not critical for use either in this preparation or in the invention in general. Any suitable strain can be used, such as, for example, strains SMD1163 and and KM71 which have the genotypes *his*4, *prB*1, *pep*4 and *his4, aox1*::SARG4. Strains GS115 and KM71 are described in Phillips patent number AU-B-63882/86 (Site selective genomic modification of yeast of the genus *Pichia).*

The plasmids were linearised with SacI and used to transform the host strain. Yeast strain GS115 was grown overnight in 200ml YEPD at 30°C in an orbital shaker. Cultures at an A600 of between 1.3-1.5 were harvested by centrifugation at 3000rpm for 5min, washed in ice cold sterile water, recentrifuged and resuspended in 8ml ice cold 1M sorbitol. 40µl cells were added to 1µg linearised DNA in an ice cold 0.2cm electroporation cuvette. After 5min on ice the cells were pulsed using the BioRad gene pulser unit at 25µF, 1.5 kV, and 400 Ohm. 1ml ice cold 1M sorbitol was added to the cuvette. 400µl of this was plated onto agarose plates and allowed to grow for 2-4 days at 30°C.

All yeast media and transformation buffers were as described in the appendix.

Transformants were collected along with the agarose overlay they had been growing in, transferred to a 50mL centrifuge tube and resusended in 50mM sodium phosphate buffer pH6. After suitable mixing and agitation to remove the cells from the agarose they were diluted and plated onto YEPD agar plates containing the antibiotic G418 at concentrations between 0 and 2000 µg/mL. Only cells in which several copies of the expresssion cassette had integrated onto the host chromosome would be able to grow on high levels of antibiotic by virtue of the several copies of the kanamycin resistance gene they would be carrying. Such cells are deemed desirable since they will also be carrying several copies of the B2M gene. Previous work has shown such multicopy integrants to be high producers under conditions were the foreign gene is expressed (Clare,J.J.,

Rayment,F.B., Ballantine,S.P., Sreekrishna,K., and Romanos,M.A., BIO/TECHNOLOGY, 9, 455-460, 1991). Plates were incubated at 30°C for 5-7 days. Colonies occuring on plates containing high concentrations of the antibiotic were then picked and streaked onto fresh MD agar plates. Single colonies were obtained after 3-4 days growth at 30°c.

Example 8 - Expression of hybrid Sendai/B2M and Influenza/B2M fusion proteins in P.pastoris

In separate experiments, single colonies of transformed strains harbouring pGC633 and (separately) pGC638 were used to inoculate 5mL of BMGC medium and the cultures were grown overnight at 30°C on an orbital shaker. This 5mL overnight culture was then used to inoculate 2L baffled shake flasks containing 50mL of the medium BMGC. After 24h growth at 30°C on an orbital incubator cells were harvested by centrifugation at 3000rpm for 5mins and resuspended in 50mL of BMMC. This induces gene expression from the AOX1 promoter. Induction was carried out by growth in the methanol containing medium at 30°C for 48 hours. After 24 hours 250 µL of sterile methanol are added to the flask to replace that lost by evaporation.

After 48 hours the culture supernatant was collected by centrifugation at 3000rpm for 5min. The supernatent was used for further analysis. Production levels from strains with the alpha factor leader sequence were estimated by SDS-PAGE to be in the order of 150mg/L.

Example 9 - Induction of Sendai NP-specific cytotoxic T-lymphocytes (CTL) by the Hybrid Sendai/B2M product of Example 7 (pGC633)

C57BL/6 (H-2^b) mice were immunised subcutaneously with either 50, 10, 2 or 0.4µg of the hbrid Sendai NP - B2M fusion protein expressed by pGC633 according to example 7, or 50µg of B2M plus 20ng of the Sendai NP epitope (FAPGNYPAL) - part of SEQ ID 9. After 6 days the spleens were removed from two

mice per group and a single cell suspension prepared which was restimulated *in vitro* with the Sendai NP peptide. After 24 hours 2% of an IL2 preparation was added. Following 7 days restimulation *in vitro* these effector cells were tested for their ability to lyse Sendai NP peptide-pulsed EL4 (H-2^b) target cells. The ratios of effector (E) to target (T) cells were 100:1, 33.3:1. 11.1:1 and 3.7:1. The results of this test were recorded as % net specific lysis, which is calculated by subtracting the lysis of non-peptide-pulsed targets from that obtained using peptide-pulsed targets.

The results are plotted in Figure 7. A peptide-specific lysis of 24.4% was seen at an ET ratio of 100:1 following immunisation with 50µg of the fusion protein. All immunisation doses of the fusion protein gave responses similar to those obtained by immunising with a mixture of B2M and Sendai NP peptide

Media recipies

BMGC

Quantities per litre:

Sodium phosphate buffer 1M,	pH6	100mL
Casamino acids (100g/L)		100mL
Yeast Nitrogen Base (13.4 g/L)		100mL
Biotin (0.5g/L)		2mL
Glycerol		10mL

Filter sterilise

BMMC

As above but replace glycerol with 5mL of methanol.

YEPD

Yeast extract	10g/L
Peptone	20g/L
Glucose	10g/L

For solid medium add 15g/L agar.

Autoclave at 121°C 15min.

CLAIMS

1. A hybrid fusion protein comprising a first antigenic amino acid sequence fused to a second amino acid sequence substantially homologous to B2M or a fragment thereof.

2. A fusion protein as claimed in claim 1 where the second amino acid sequence is that of naturally occurring B2M.

3. A fusion protein as claimed in claim 1 or claim 2 where the antigenic sequence corresponds to a sequence derived from or associated with an aetiological agent or a tumour.

4 A fusion protein as claimed in claim 3 where the aetiological agent is a microorganism such as a virus, bacterium, fungus or parasite.

5 A fusion protein as claimed in claim 4 where the virus is a retrovirus, such as HIV-1, HIV-2, HTLV-I, HTLV-II, HTLV-III, SIV, BIV, LAV, ELAV, CIAV, murine leukaemia virus, Moloney murine leukaemia virus, and feline leukaemia virus; an orthomyxovirus, such as influenza A or B; a paramyxovirus, such as parainfluenza virus, mumps, measles, RSV and Sendai virus; a papovavirus, such as HPV; an arenavirus, such as LCMV of humans or mice; a hepadnavirus, such as Hepatitis B virus; a herpes virus, such as HSV, VZV, CMV, or EBV.

6 A fusion protein as claimed in claim 4 where the antigenic sequence is derived from a bacterium, such as of the genus *Neisseria*, *Campylobacter*, *Bordetella*, *Listeria*, *Mycobacteria* or *Leishmania*, or a parasite, such as from the genus *Trypanosoma, Schizosoma, Plasmodium*, especially *P. falciparum*, or from a fungus, such as from the genus *Candida, Aspergillus, Cryptococcus, Histoplasma* or *Blastomyces*.

7 A fusion protein as claimed in claim 4 where the antigenic sequence is a proteinaceous human tumour antigen, such as a melanoma-associated

antigen, or an epithelial-tumour associated antigen such as from breast or colon carcinoma.

8 A fusion protein as claimed in claim 4 where the antigenic sequence is an epitope from:

1) HIV (particularly HIV-1) gp120,
2) HIV (particularly HIV-1) p24
3) VZV gpI, gpII and gpIII
4) LCMV nucleoprotein,
5) Influenza virus nucleoprotein,
6) HPV L1 and L2 proteins,
7) Human papilloma virus E5 and E7
8) Malaria CSP or RESA antigens,
9) Mycobacterium p6,
10) GA 733-2 epithelial tumour-associated antigen,
11) MUC-1 repeat sequence from epithelial tumour-associated antigen,
12) Melanoma MZ2-E antigens
13) Melanoma p97 associated antigen,

9 A fusion protein as claimed in claim 4 where the antigenic sequence is an epitope from the third variable domain of an envelope protein of a lentivirus.

10. A fusion protein as claimed in any of the preceding claims wherein the antigenic sequence is fused to the B2M sequence via a linker sequence.

11. Nucleic acid coding for a fusion protein as claimed in any one of claims 1 to 10.

12. A vector including nucleic acid as claimed in claim 11.

13. A host cell carrying a vector as claimed in claim 12.

14 A host cell as claimed in claim 13 where the host cell is *E. coli*

15. A host cell as claimed in claim 13 where the host cell is a yeast cell such as *Saccharomyces cerevisiae* or *Pichia pastoris*

16. A host cell as claimed in claim 13 where the host cell is an insect cell such as *Spodoptera frugiperda* SF9, or mammalian cells including Chinese hamster ovary (CHO) cells, mouse myeloma cell lines such as P3X63-Ag8.653, COS cells, HeLa cells, BHK cells, melanoma cell lines such as the Bowes cell line, mouse L cells, human hepatoma cell lines such as Hep G2, mouse fibroblasts and mouse NIH 3T3 cells.

17 A pharmaceutical or veterinary formulation comprising a B2M fusion protein as claimed in any one of claims 1-10 and a pharmaceutically or veterinarily acceptable carrier.

18 A pharmaceutical or veterinary formulation as claimed in claim 17 comprising in addition a subunit vaccines designed to induce good neutralising antibody responses.

19. A B2M fusion protein as claimed in any one of claims 1-10 for use as a prophylactic or immunotherapeutic vaccine.

20. The use of a B2M fusion protein as claimed in any one of claims 1-10 in the preparation of a prophylactic or immunotherapeutic vaccine

21 A method of producing a B2M fusion protein as claimed in any of claims 1-10 by cultivating a methylotropic yeast harbouring an expression vector comprising DNA encoding the relevant fusion protein, and recovering the expressed fusion protein.

22 A method as claimed in claim 21 where the yeast is *Pichia pastoris*.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/00755

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/62 C12N5/10 C12N1/21 C12N1/19 A61K39/21
A61K39/145 C07K13/00 A61K39/155 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 267, no. 34 , 1992 , BALTIMORE US pages 24223 - 24229 F. GODEAU ETAL 'Purification and ligand binding of a soluble class I major histocompatability complex molecule consisting of the first three domains of H-2Kd fused to beta-2-microglobulin expressed in the baculovirus-insect cell system' see the whole document --- -/--	1,2, 10-13,16

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
23 August 1994	05 -09- 1994

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88 , January 1991 , WASHINGTON US pages 301 - 304 K. ROCK ET AL 'Reassociation with beta-2-microglobulin is necessary for Db class I major histocompatability complex binding of an exogenous influenza peptide' see the whole document	5,8, 17-20
A	JOURNAL OF IMMUNOLOGY. vol. 150, no. 4 , 15 February 1993 , BALTIMORE US pages 1244 - 1252 K.ROCK ET AL 'Peptide-priming of cytolytic T cell immunity in vivo using beta-2-microglobulin as an adjuvant' see the whole document	5,17-20
A	GENE. vol. 83 , 1989 , AMSTERDAM NL pages 117 - 124 K. PARKER AND D. WILEY 'Overexpression of native human beta-2-microglobulin in Escherichia coli and its purification' see the whole document	1-14

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